ACS APPLIED MATERIALS & INTERFACES

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Functional Polysaccharide Conjugates for the Preparation of Microarrays

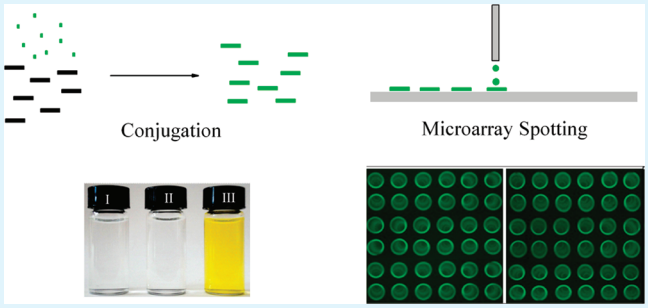
Tamilselvan Mohan,[†] Rupert Kargl,*[,‡] Stefan Köstler,[§] Aleš Doliška,[†] Gerald Findenig,[‡] Volker Ribitsch,[†,§,⊥] and Karin Stana-Kleinschek*[,†,⊥]

[†]Laboratory for Characterization and Processing of Polymers, Faculty of Mechanical Engineering, University of Maribor, Smetanova 17, SI-2000 Maribor, Slovenia

[‡]Institute of Chemistry, Karl-Franzens-University Graz, Heinrichstraße 28/III, AT-8010 Graz, Austria

[§]Joanneum Research, Materials, Institute for Surface Technologies and Photonics, Steyrergasse 17-19, AT-8010 Graz, Austria

ABSTRACT: A method for the immobilization of functional molecules on cellulose surfaces was developed. The irreversible deposition of the water-soluble polyelectrolyte carboxymethyl cellulose (CMC) on solid cellulose surfaces was used as a basis for this immobilization. CMC was modified using amino-fluorescein (AMF) as a model compound for a functional molecule. The carbodiimide mediated coupling efficiency of AMF to CMC was studied in detail, and the functional conjugates were isolated. A quartz crystal microbalance with dissipation was employed to study the immobilization of the functionalized CMC onto cellulose model films in situ. The influence of the carbodiimide concentration, the degree of substitution, and the molecular weight of CMC on the immobilization process was investigated. Atomic force microscopy was used to characterize the changes in the surface morphology of the modified cellulose films. Finally, microspotted arrays of AMF-CMC conjugates were prepared with the knowledge obtained from the basic interaction studies. The successful deposition of AMF-CMC conjugates onto cellulose surfaces was proven by fluorescence microscopy. The conjugation of functional molecules to CMC and the subsequent deposition of these products on cellulose can be seen as a versatile method to immobilize these molecules for applications in the field of microarrays and other sensor surfaces. It offers the possibility to introduce new properties on a variety of cellulosic materials.



KEYWORDS: *carboxymethyl cellulose, microarrays, quartz crystal microbalance, carbodiimide coupling, aminofluorescein*

INTRODUCTION

Surface functionalization with biomolecules by either covalent attachment or adsorptive interaction is of great interest if one aims to introduce new properties onto these surfaces. Especially for the manufacturing of biosensors, surface functionalization with receptor molecules is one of the crucial steps.[1,2] Until now, there is a great variety of surface modification and immobilization methods. For the sensing of DNA or for protein microarray technology, covalent immobilization of the species of interest is often performed using surfaces bearing aldehydes or epoxides as reactive groups.[3,4] The immobilization efficiency and irreversibility on these surfaces is high. Nevertheless, the surfaces have a reduced shelf life, and additional blocking steps of free reactive groups are often required. Another encountered problem on sensors surfaces is the nonspecific interaction of biomolecules with the substrates during the immobilization or detection process.[5] These interactions can reduce the sensitivity and the limit of quantification. To overcome these limitations, substrates without reactive groups or with low unspecific binding can be used.[6]

As a typical example for surfaces without reactive groups, thin films of cellulose derivatives are used for the adsorptive immobilization of biomolecules. Besides their three-dimensional structure of for instance nitro cellulose, these surfaces do not bear easily reacting groups, provide a high surface area, and are a stable environment for protein attachments.[7] It was shown recently that besides nitro cellulose also pure cellulose surfaces can be used to immobilize biological analytes or receptor molecules.[8] Pure cellulose thin films provide low unspecific binding but a high surface area for the effective immobilization of receptors and the detection of analytes.[9,10] Especially pure cellulose surfaces seem to prevent the unspecific binding of biomolecules through their strong surface hydration.[11] In contrast, it is known that several polysaccharides tend to adsorb on cellulose surfaces with a relatively high specificity. This binding is often attributed to the structural similarity of soluble polysaccharides and solid cellulose surfaces.[12−14] Especially the water-soluble cellulose derivative carboxymethyl cellulose

Received: February 29, 2012
Accepted: April 19, 2012
Published: April 19, 2012

(CMC) is known to irreversibly attach to a cellulose surface under defined conditions.[15,16] This fact and the aforementioned advantageous surface properties of cellulose can be exploited for the conjugation of functional molecules to solid cellulose. Polysaccharides with carboxylic acid moieties as in CMC can be conjugated to amino-group containing functional molecules with the well established carbodiimide chemistry.[17−20] This reaction in combination with the deposition of CMC on cellulose can offer the possibility to immobilize functional molecules on solid supports.

The work presented here aims at conjugating aminofluorescein (AMF) with CMC and immobilizing these conjugates onto a solid cellulose thin film. The immobilization should take advantage of the relatively specific interaction of CMC with cellulose surfaces and the low unspecific binding of the cellulose thin films. Aminofluorescein was chosen as a model compound for the functional molecule because it bears primary amine moieties similar to many proteins. The conjugation of AMF to CMC via an amide bond was performed using carbodiimide chemistry. The coupling efficiency was studied with UV−vis spectroscopy of isolated, purified conjugates. A quartz crystal microbalance with dissipation (QCM-D) and a microarray spotting device were further used to study the deposition of the conjugates onto cellulose coated microscope slides. The influence of the EDC concentration and the molecular weight and degree of substitution on the amount of irreversibly deposited conjugates was studied. Atomic force microscopy was further applied to investigate the topography of the modified surfaces. The study should show the application potential of the immobilization method in the field of microarray and biosensor technology.

1. EXPERIMENTAL SECTION

1.1. Materials. Trimethylsilyl cellulose (TMSC) with a degree of substitution (DS_{TMS}) of 2.55 (kindly provided by the Centre of Excellence for Polysaccharide Research, University of Jena) was used as a starting material for cellulose surface preparation and synthesized according to a published literature procedure.[21] Four different sodium salts of carboxymethyl cellulose (CMC) with varying DS_{COONa} and molecular weights (M_w) were purchased from Sigma-Aldrich, Austria (CMC1: DS 0.7, $M_w \approx 90$ kDa; CMC2: DS 0.7, $M_w \approx 250$ kDa; CMC3 DS 0.9, $M_w \approx 250$ kDa; CMC4: DS 1.2, $M_w \approx 250$ kDa). Toluene (99.9%), 2-butanone (≥99%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), and 5-aminofluorescein (AMF) were from Sigma-Aldrich, Austria. All chemicals were used as received. Sterilized cyclic olefine polymer slides (COP; Zeonor1060R), 75 × 25 mm^2, were kindly provided by SonyDADC, Austria. Five MHz QCM-D sensor crystals with gold electrode surfaces (QSX303) were purchased from LOT-Oriel (Germany). Milli-Q water from a Millipore (MA, USA) water purification system (resistivity ≥18.2 MΩ cm, pH 6.8) was used for the preparation of all aqueous solutions throughout the whole work.

1.2. Cellulose Film Preparation. For QCM-D measurement, sensor crystals with a gold surface were used as substrates for the spin coating with TMSC. The crystals were soaked into a mixture of H_2O/H_2O_2 (30 wt %)/NH_4OH (5:1:1; v/v/v) for 10 min at 70 °C. Afterward they were immersed into a "piranha" solution (H_2O_2 (30 wt %)/H_2SO_4 (98 wt %) (1:3; v/v)) for 60 s, rinsed with water again, and finally blow dried with nitrogen gas. (*Caution*: piranha solution is highly reactive, corrosive and potentially explosive. During preparation, temperatures above 100 °C can be reached easily). For spin coating of TMSC with a spin coater (Polos MCD wafer spinner, APT corporation, Germany), 50 μL of TMSC (1 wt % solution in toluene) was deposited on the static QCM-D Au-crystal which was then rotated for 60 s with a spinning speed of 4000 rpm and an acceleration of 2500 rpm s^{-1}. For spin coating of TMSC on COP, 2 mL of TMSC solution (0.5 wt % dissolved in 2-butanone by heating to 40 °C) was deposited on the static substrate. Spin coating was performed at 4000 rpm for 60 s with an acceleration of 2500 rpm s^{-1}. The TMSC coated QCM-D Au-crystals were placed into a 20 mL polystyrene Petri dish containing 2 mL of 10 wt % HCl. The dish was covered with its cap, and the films were exposed to the vapor phase of HCl for 10 min. The details of this procedure are published elsewhere.[22,23] For TMSC coated on COP, the regeneration was performed with a volume of 200 mL of 10 wt % HCl. The HCl vapor was stabilized for 1 h in a 300 mL vacuum desiccator. The TMSC spin coated COP slide was regenerated for 10 min by placing it into this desiccator.

1.3. Conjugation of Aminofluorescein and CMC. Four different commercial CMCs were used for the conjugation with 5-aminofluorescein (AMF). The CMCs (4 mg mL^{-1}) were dissolved in water and stirred overnight. The CMC solutions were filtered using 0.45 μm PVDF (polyvinylidene fluoride) syringe filters. EDC solutions were prepared at concentrations of 37.2, 18.6, and 3.7 mg mL^{-1} in water. AMF solutions (0.676 mg mL^{-1}) were prepared in water. Complete dissolution of AMF was achieved at pH 7 with 0.1 M NaOH. Mixtures of CMC, EDC, and AMF solutions were prepared in a ratio of 2:1:1 (v/v/v) for each concentration of EDC. As a result, the final CMC concentration (2 mg mL^{-1}) and AMF concentration (0.169 mg mL^{-1}) were constant, whereas the concentration of EDC was varied (9.3, 4.7, and 0.93 mg mL^{-1}). All mixtures and the resulting products of AMF, CMC, and EDC are denoted as AMF-CMC*x*-(*y*), where *x* describes the type of CMC and *y* describes the concentration of EDC in the reaction mixture in mg mL^{-1}. Mixtures and products made without the addition of AMF are described as CMC*x*-(*y*). The reaction time for all mixtures was set to 3 h at room temperature in a dark place. After that, 40 mL of each AMF-CMC-EDC mixture was dialyzed against Milli-Q water using dialysis membranes with 14 kDa M_W cutoff (Roth, Germany). The permeate was exchanged every 4 h, and the dialysis was performed for 4 d. The dialyzed solutions were lyophilized for 4 d at 10^{-3} mbar and −25 °C. During dialysis and lyophilization, all samples were stored under the exclusion of light. For the interaction studies of the isolated and purified products with cellulose thin films, the products were dissolved in pure water at a concentration of 2 mg mL^{-1}, stirred for 120 min, and filtrated with a 0.45 μm PVDF syringe filter.

For the in situ interaction studies of the AMF-CMC conjugates with cellulose thin films, the reaction mixtures of AMF-CMC-EDC were directly applied to the QCM-D modules after a reaction time of 3 h without prior isolation or purification. For microarray spotting, COP slides spin coated with cellulose were used. The spotting solutions were prepared in the same way as the solutions for the in situ QCM-D measurements. The mixtures were allowed to react for 3 h under light exclusion at room temperature, diluted with water in a ratio of 1:1 (v/v), and immediately spotted onto the cellulose surfaces using a microarray spotting device described in section 1.8.

1.4. UV−Vis Absorption Spectroscopy. Determination of the UV−vis absorption spectra was performed with a Carry 50 spectrophotometer at room temperature. The purified, isolated products AMF-CMC1-(0), AMF-CMC1-(0.93), AMF-CMC1-(4.7), and AMF-CMC1-(9.3) were dissolved at a concentration of 0.2 mg mL^{-1}. All samples were prepared in Milli-Q water at neutral pH. The amount of AMF in solutions of purified AMF-CMC conjugates was calculated from a linear calibration curve of different AMF concentrations. Pure AMF solutions (0.01, 0.005, 0.0025, and 0.00125 mg mL^{-1}) were prepared in water at neutral pH and measured without further purification. The degree of substitution for AMF-CMC conjugates was calculated from the amount of AMF that was found in the AMF-CMC conjugation products.

1.5. FTIR Spectroscopy. Infrared absorption spectra of purified, isolated conjugates were recorded for the products CMC1, CMC1-(9.3), and AMF-CMC1-(9.3) using a PerkinElmer Spectrum GX Series-73565 FTIR-spectrometer at a scan range from 4000 to 650 cm^{-1}. 32 scans were performed for all samples with a resolution of 4 cm^{-1}. All samples were measured as a KBr pellet.

1.6. Potentiometric Charge Titration. The potentiometric charge titration of the sample solutions was carried out with a two-

buret instrument from Mettler Toledo T70, under inert atmosphere (nitrogen gas bubbling). The isolated purified samples of CMC1, CMC1-(9.3), and AMF-CMC1-(9.3) (1.5 mg mL^{-1}) were titrated in a forward and back manner between the initial pH 2 to the preset pH 11. All measurements were repeated twice. A detailed description of the potentiometric charge titration method can be found elsewhere.[24] The amounts of carboxylic groups present in the products were expressed in mmol g^{-1} sample.

1.7. Quartz Crystal Microbalance with Dissipation Measurements (QCM-D). A QCM-D E4 from Q-Sense AB, Gothenburg, Sweden was used for interaction studies of the isolated and purified AMF-CMC conjugates and the AMF-CMC-EDC reaction mixtures with solid cellulose surfaces. The instrument determines changes in frequency (f) and dissipation (D) of an oscillating quartz crystal. Deposition of mass or changes in the rigidity of material on the crystals surface can be detected. Negative frequency shifts (Δf) indicate a deposition of mass whereas positive dissipation shifts (ΔD) are caused by a reduced rigidity of the coating. The reduced rigidity is often a result of incorporated solvent molecules and extended swelling. A detailed description of the QCM-D method can be found elsewhere.[25,26]

The temperature during all measurements was set to 21 ± 0.1 °C. The cellulose coated gold crystals were assembled in the QCM-D chamber. At each run, Milli-Q water (flow rate: 0.1 mL min^{-1}) was pumped through the chamber until a stable baseline frequency was obtained. All frequency and dissipation values were set to zero, and the measurement was started.

After 5 min, the isolated and purified conjugates or the AMF-CMC-EDC reaction mixtures were introduced into the QCM-D modules (flow rate: 0.1 mL min^{-1}). All experiments were conducted in a continuous flow mode in which the cellulose surfaces were constantly exposed to the solutions. The surfaces were exposed for at least 90 min until only minor changes in frequency and dissipation (Δf_3 and ΔD_3) of the third overtone of the oscillation were observed. After that, the surfaces were rinsed with Milli-Q water to remove unbound material. The crystals were taken out of the chamber, blow dried with nitrogen gas, and stored under the exclusion of light.

The pH dependent stability of the adsorbed CMC layers on the cellulose model films was determined in separate experiments by incubation with isolated purified products of AMF-CMC1-(9.3) or in situ immobilized AMF-CMC1-(9.3). After incubation with the conjugation products for 90 min, the films were rinsed with pure water at pH 7 for 8 h followed by rinsing with water at pH 3, 7, or 9 (adjusted with 0.1 M NaOH or HCl) for 8 h. This was followed again by rinsing with pure water for 8 h.

In addition, the immobilized layers were subjected to rinsing with a washing buffer (15 mL of SSC (sodium saline citrate) 20× concentrate: 0.3 M sodium citrate, pH 7.0, 3 M NaCl, Sigma-Aldrich, product no. S6639, 600 μL of surfactant Tween 20 (Merck, Germany) and 584.4 mL of pure water) for 24 h.

1.8. Microarray Spotting and Fluorescence Microscopy. Microarray spotting was performed using a noncontact microarray spotter (sciFLEXARRAYER S3, Scienion, Germany). Spotting was performed with 350 pL per drop at 24 °C and a relative humidity of 70%. After the spotting run, the surfaces were stored at room temperature under the exclusion of light for 30 min. Finally the surfaces were extensively rinsed with water and blow dried with nitrogen gas. The spots were analyzed by fluorescence microscopy using an Olympus BX51 microscope with blue excitation and green emission (Olympus U-MWIB filter cube). The light source for excitation was an Hg arc lamp.

1.9. Atomic Force Microscopy (AFM). Topographical features of cellulose surfaces on the QCM-D crystals that were modified with AMF-CMC conjugates were characterized by AFM in the intermittent contact mode with an Agilent 5500 AFM multimode scanning probe microscope (Digital Instruments, Santa Barbara, CA). Silicon cantilevers (ATEC-NC-20, Nanosensors, Germany) with a resonance frequency of 210–490 kHz and a force constant of 12–110 N m^{-1} were used. The scanned image size was 1 × 1 μm^2. All measurements were performed at ambient temperature in air.

2. RESULTS AND DISCUSSION

2.1. UV–Vis Absorption Spectroscopy. The UV–vis absorption spectra of purified, isolated AMF-CMC conjugates reveal several interesting details (Figure 1). The sample AMF-

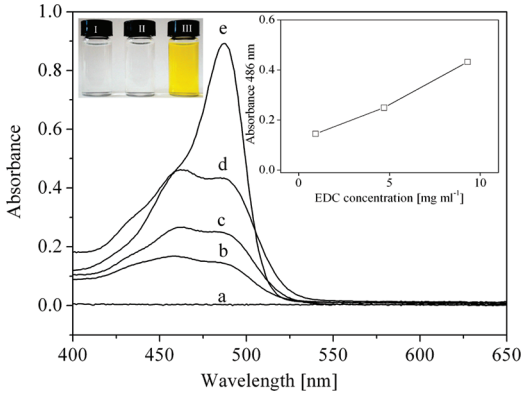


Figure 1. Absorption spectra of isolated and purified (a) AMF-CMC1-(0), (b) AMF-CMC1-(0.93), (c) AMF-CMC1-(4.7), (d) AMF-CMC1-(9.3), and (e) pure AMF. Inset: absorption at 486 nm as a function of the EDC concentration. Images of aqueous solutions of isolated and purified (I) CMC1, (II) AMF-CMC1-(0), and (III) AMF-CMC1-(9.3) are shown.

CMC1-(0), which did not contain any EDC during the reaction, does not show any absorbance of visible light. This proves that AMF was removed during the dialysis and was not bound to the CMC backbone. The samples with increasing EDC concentrations AMF-CMC1-(0.93) to AMF-CMC1-(9.3) show absorption peaks at 462 and 486 nm which are due to bound AMF that cannot be removed by dialysis. Similar absorption peaks are observed for pure AMF solutions. Nevertheless differences in the form of the spectra can be most likely attributed to the binding of AMF to the CMC backbone which results in a different chemical environment. The inset in Figure 1 depicts a linear relation between the absorbance at 486 nm and the EDC concentration during the coupling reaction. It can be clearly seen that the AMF coupling density increases when more EDC is used. Obviously higher EDC concentrations improve the activation efficiency of carboxylic groups for the subsequent coupling with AMF. The successful coupling of AMF to CMC can already be visualized by the naked eye as shown in the left inset of Figure 1. Solutions of CMC1 (I), AMF-CMC1-(0) (II), and AMF-CMC1-(9.3) (III) each at a concentration of 0.2 mg mL^{-1} are shown. The CMC1 and AMF-CMC1-(0) solutions are clear and colorless in appearance, whereas AMF-CMC1-(9.3) exhibits a yellow color. This proves that AMF is bound to CMC when EDC is used as a conjugation agent. From the absorption of the AMF-CMC conjugates at 486 nm, one can estimate the amount of AMF present in the AMF-CMC conjugate solutions. For the sample AMF-CMC1-(9.3), this is 2.4 wt % AMF. If one assumes that all AMF molecules are covalently bound to CMC, this corresponds to a DS_{AMF} of 0.02. On average, every 50th monomeric unit of CMC is substituted by AMF. This is a reasonable value if one considers that the initial monomer ratio of CMC1 to AMF in the reaction mixture was 11:1. These results demonstrate that EDC coupling can successfully be used to fluorescent label carboxylic moieties within a polysaccharide backbone. Even though the labeling is not quantitative, it is a facile single step procedure with

acceptable yields. Fluorescein was used as a model substance to prove the coupling to CMC and to subsequently immobilize the conjugates on cellulose surfaces. Nevertheless the coupling reaction is obviously not limited to fluorescent dyes since any primary amino group containing molecule can be linked to CMC.

The activation mechanism of carboxylic groups with EDC in aqueous media is rather complex. EDC can react with carboxylic groups of CMC molecules to form the unstable intermediate O-acylisourea. In the presence of primary amines, the O-acylisourea formation is followed by the formation of an amide bond which can occur even at neutral pH.[27] It is worth noting that all reactions in this work were performed at neutral pH. In addition to this, the side products N-acylurea and acid anhydride intermediates can be formed during the reaction.[28,29] This can lead to unwanted derivatization of CMC. EDC is also known to promote cross-linking of water-soluble polysaccharides such as CMC or hyaluronic acid, through inter- or intramolecular ester bond formation between carboxylic and hydroxyl groups.[17] The concentration and accessibility of carboxylic groups is then reduced, before an amide bond formation can take place. It can come to the formation of a high molecular weight cross-linked CMC derivative, which most likely contains bound and free AMF. In our cases, the isolated and purified products derived from this reaction are water-soluble and their preparation is fast, reproducible, and simple. With further optimization steps, one could use this method for the conjugation of any primary amine-containing functional molecule to the backbone of water-soluble, carboxylic group containing polymers. The isolation and storage of CMC conjugated functional molecules for further application could be possible with this method.

2.2. FTIR Spectroscopy and Charge Titration. The infrared (IR) absorption spectra of purified, isolated samples of CMC1, CMC1-(9.3), and AMF-CMC1-(9.3) are shown in Figure 2. The IR spectrum of (a) CMC1 shows O−H, C−H, and C−O−C stretching vibrations in the range of 3000−3690 and at 2896, 1017, and 1046 cm^{-1} and carbonyl vibrations at 1590 cm^{-1}. These absorption bands were also observed for the samples (b) CMC1-(9.3) and (c) AMF-CMC1-(9.3).

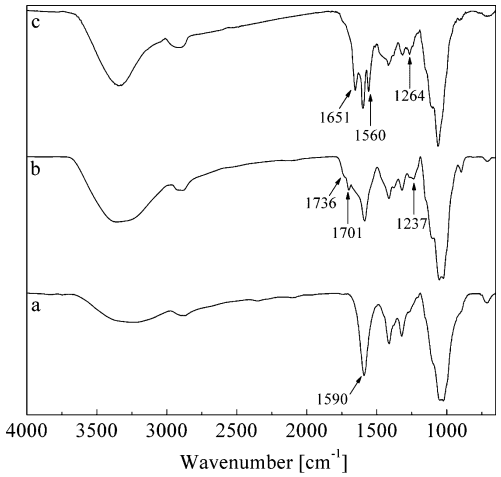


Figure 2. FT-IR absorption spectra of the isolated and purified products of (a) CMC1, (b) CMC1-(9.3), and (c) AMF-CMC1-(9.3).

All these peaks are typical for carboxymethylated cellulose.[30,31] In contrast, AMF-CMC1-(9.3) shows two new peaks at 1651 cm^{-1} (N−H amide I band) and 1560 cm^{-1} (N−H secondary amide II band)[18] This confirms that AMF is covalently bound to CMC1 via the formation of an amide bond.[32,33] In addition to this, bands at 1475 and 1264 cm^{-1} for C═C vibrations of aromatic rings from AMF and for C−N stretching are present.[34] The IR spectra show that CMC1-(9.3) is not completely pure CMC. The formation of O-acylisourea or N-acylurea derivatives during the activation with EDC is likely. The bands at 1736 cm^{-1} (free and hydrogen bonded carboxylic group), 1701 cm^{-1} (carbonyl group of the ester intermediate), and 1237 cm^{-1} (carbonyl stretching) can be attributed to the ester intermediates of activated CMC.[18,31] Potentiometric charge titration of the isolated purified products showed a reduction of the amounts of carboxylic groups (CMC1: 3.32 ± 0.03 mmol g^{-1}; CMC1-(9.3): 1.26 ± 0.02 mmol g^{-1}; AMF-CMC1-(9.3): 1.21 ± 0.03 mmol g^{-1}) after reaction with EDC or EDC AMF. Cross-linking and side-reactions can be considered as the main reasons for that reduction.

2.3. Interaction of Isolated, Purified Products with Cellulose Surfaces. The isolated and purified AMF-CMC conjugates (0.2 mg mL^{-1}) from section 2.1 were used for interaction studies with solid cellulose thin films in the QCM-D. Figure 3 shows the QCM-D frequency (Δf_3) and dissipation (ΔD_3) shifts for the products (a) AMF-CMC1-(0), (b) CMC1-(9.3), and (c) AMF-CMC1-(9.3). The product AMF-CMC1-(0) does not show any significant irreversible deposition on cellulose thin films at neutral pH after rinsing with pure water. Frequency and dissipation shifts reach almost the baseline level after rinsing with water. This is in agreement with other studies where low rates of deposition without the addition of electrolytes or changes in the pH value were observed.[16] The isolated carboxymethyl cellulose which was reacted solely with EDC for 3 h ((b) CMC1-(9.3)) showed a different behavior. The deposition of this product on cellulose is higher, which can be attributed to a possible cross-linking with EDC and the reduction of negative charges on the CMC backbone. This cross-linking increases the molecular weight and reduces the

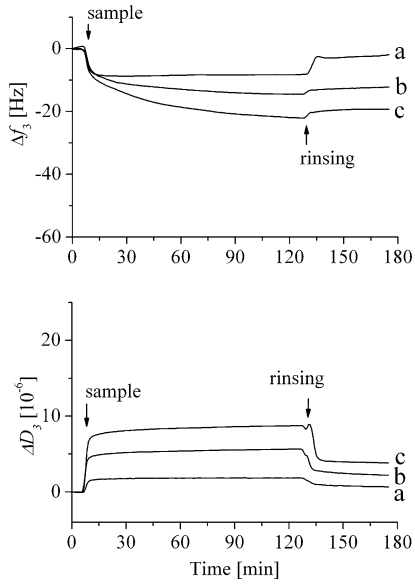


Figure 3. Changes in frequency (Δf_3) and dissipation (ΔD_3) of the isolated, purified products of (a) AMF-CMC1-(0), (b) CMC1-(9.3), and (c) AMF-CMC1-(9.3) incubated onto cellulose surfaces.

solubility of CMC whereas a reduction of negative charges reduces the electrostatic repulsion. Increased dissipation values indicate a swollen deposited layer on the cellulose surface. The conjugates of carboxymethyl cellulose and aminofluorescein ((c) AMF-CMC1-(9.3)), which definitely contain bound fluorescein, are irreversibly deposited on the cellulose surfaces at higher rates, indicated by lower frequency and higher dissipation values after rinsing. Conjugation of AMF to CMC seems to cause a higher deposition rate on the cellulose surfaces. These results show that it is possible to immobilize isolated, functionalized CMC molecules on cellulose solid supports at neutral pH and without the addition of electrolytes except EDC. All solutions of isolated purified conjugation products had a neutral pH which is favorable if pH sensitive biomolecules have to be immobilized.

Isolation and purification of derivatized CMC can be in addition favorable in terms of the general applicability of the immobilization method. For the application in microarray spotting, an in situ immobilization without further purification is more advantageous. Within the next sections, we therefore investigated the in situ interaction of nonisolated conjugates of AMF-CMC with solid cellulose.

2.4. In Situ Interaction of AMF-CMC Reaction Mixtures with Cellulose Surfaces. *2.4.1. Effect of the EDC Concentration.* The AMF-CMC1 reaction mixtures were applied to cellulose model films in the QCM-D modules without any additional purification after 3 h of reaction. An application without purification was chosen, since it mimics the microarray spotting conditions described in section 2.6. In microarray spotting, it is advantageous to simply mix amine groups containing functional molecules with CMC and EDC and to spot them without any further treatment onto a substrate. Figure 4 shows the changes in frequency (Δf_3) and dissipation (ΔD_3) for (a) CMC1, (b) CMC1-(9.3), (c) AMF-CMC1-(0.93), and (d) AMF-CMC1-(9.3) on a cellulose model film. The maximum Δf_3 and ΔD_3 before rinsing were found for the highest EDC concentration when AMF was present (d). Pure CMC1 showed lower rates of immobilization before rinsing, even if EDC was present (a, b). After approximately

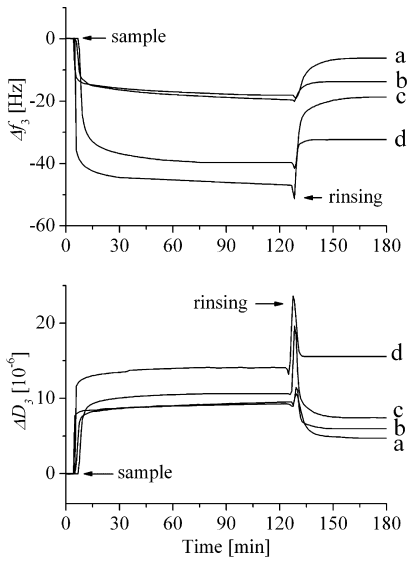


Figure 4. Changes in frequency (Δf_3) and dissipation (ΔD_3) of (a) CMC1, (b) CMC1-(9.3), (c) AMF-CMC1-(0.93), and (d) AMF-CMC1-(9.3) reaction mixtures incubated onto cellulose surfaces.

120 min, the surfaces were rinsed with pure water, which led to an increase in f_3 for all reaction mixtures. Dissipation values after rinsing decreased for the mixtures a, b, and c but increased for mixture d.

There are several possibilities to explain these phenomena. Elevated EDC concentrations can, besides cross-linking, lead to an increased activation and derivatization of CMC.[27] The reaction of CMC with EDC leads to a reduction of the amount of carboxylic groups as was shown by potentiometric charge titration. This reduces the negative charge of the polymer and introduces tertiary amines from EDC. These positive charges can increase the immobilization of the polymer on the surface via the formation of complexes between positive and negative charges of CMC and derivatized CMC. These complexes can further be formed between CMC molecules in solution which would increase the size of the molecules and lead to reduced solubility and extended immobilization on the surface. The binding of activated CMC is enhanced, when more carboxylic groups are derivatized and electrostatic repulsion is screened. This screening also occurs with EDC since this molecule already increases the ionic strength of the solution. The influence of the pH value on the deposition was not studied in detail since all solutions used in this work had a neutral pH value.

As was shown in section 2.1, more AMF is bound to CMC when more EDC is present. This can also lead to higher rates of immobilization since it is known that hydrophobically modified polysaccharides tend to bind spontaneously to cellulose thin films.[35] AMF can be partially seen as such a hydrophobic molecule. This effect becomes obvious if one compares the deposition rates of (b) CMC1-(9.3) and (d) AMF-CMC1-(9.3) where more material is deposited in the presence of AMF. The dissipation values follow the same trend as the frequency values. Higher frequency shifts are also reflected in higher dissipation during application of the reaction mixture. After rinsing with pure water, the dissipation only increases for (d) AMF-CMC1-(9.3) which can be interpreted as an extended swelling of the immobilized layer. It should be noted that the observed Δf_3 and ΔD_3 are always a combination of immobilized conjugate and water bound to the polymer.[36]

With increasing immobilization of AMF-CMC conjugates, the hydration capacity of the adsorbed layer increases and the system behaves like a hydrogel on the surface. When pure QCM-D Au crystals without any cellulose coating are incubated with AMF-CMC1-(9.3), no significant amounts of polymer conjugates are immobilized. In contrast, relatively high amounts are immobilized on cellulose thin films. Even though the real driving force behind the interaction of AMF-CMC with cellulose is unclear, one can assume also a contribution of a selective CMC−cellulose interaction.[14,16,37] Therefore the CMC conjugation is a reliable method to immobilize functional molecules on cellulose surfaces. The in situ immobilization of the unpurified reaction mixtures is fast and easy to perform and therefore has several advantages compared to the immobilization of the isolated purified CMC conjugation products.

2.4.2. Effect of the Degree of Substitution on the Immobilization of AMF-CMC-EDC Reaction Mixtures. Besides the effect of the EDC concentration on the immobilization and conjugation efficiency, the influence of different degrees of substitution of carboxylic groups was studied. The highest concentration of EDC in the reaction mixture (9.3 mg mL^{-1}) was chosen since it allowed the immobilization of the highest amounts of AMF-CMC1 as was shown in section 2.4.1. In

order to investigate the influence of EDC and AMF on the deposition, mixtures with and without AMF were prepared.

The frequency and dissipation values of the AMF free reaction mixtures of (a) CMC2-(9.3), (b) CMC3-(9.3), and (c) CMC4-(9.3), (M_w:~250 kDa), which were applied on cellulose films after 3 h of reaction without further purification, are shown in Figure 5. The lower the degree of substitution, the

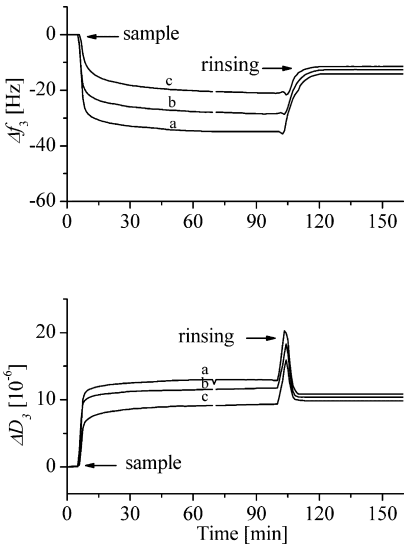


Figure 5. Changes in frequency (Δf_3) and dissipation (ΔD_3) of reaction mixtures of (a) CMC2-(9.3), (b) CMC3-(9.3), and (c) CMC4-(9.3), immobilized onto cellulose surfaces.

higher the frequency shifts after introduction of the CMC-EDC mixtures. Pure CMC solutions lead only to minor frequency and dissipation shifts after rinsing and were therefore not included in Figure 5. After rinsing with water, the final frequency shifts do not differ significantly for all three CMC-EDC mixtures. However, it is possible to immobilize sufficient amounts of CMC, which is most likely an effect of reduced charges of the CMC, cross-linking, and an increased ionic strength of the solutions. Furthermore a covalent binding of the activated CMC to the cellulose surface can be a possible explanation of the observed deposition.

In comparison, Figure 6 shows the frequency and dissipation values of the AMF-CMC-EDC containing reaction mixtures of (a) AMF-CMC2-(9.3), (b) AMF-CMC3-(9.3), and (c) AMF-CMC4-(9.3) (M_w: ~250 kDa) which were applied on cellulose films after 3 h of reaction without further purification. Even in this case, higher frequency and dissipation shifts are observed at a lower degree of substitution after introduction of the reaction mixtures into the QCM-D cells. After rinsing with water, the immobilization efficiency is strongly influenced by the degree of substitution of carboxymethyl groups in the presence of AMF. At a lower DS (CMC2, DS_{COONa} 0.7), more material is immobilized on the cellulose surface. A reason for this can be found in the solubility and efficiency of EDC activation and AMF derivatization. A higher degree of activation is easier to achieve at lower amounts of carboxylic groups. This activation can again lead to the effects of enhanced immobilization which were discussed in section 2.4.1. The differences in the immobilized amounts depend on the quantity of coupled AMF, the amount of cross-linked products, and the maximum number of activated carboxyl groups in the polymer chain. Those effects together cause higher deposition rates at a lower

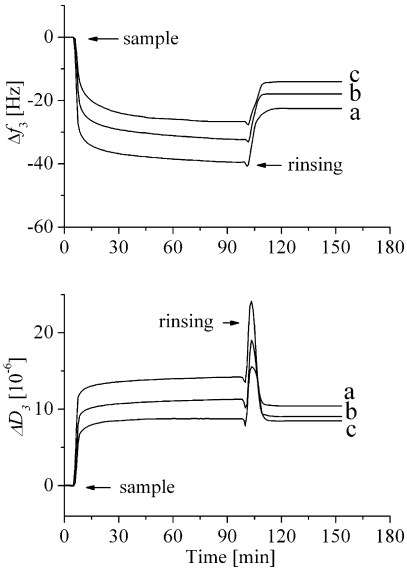


Figure 6. Changes in frequency (Δf_3) and dissipation (ΔD_3) of reaction mixtures of (a) AMF-CMC2-(9.3), (b) AMF-CMC3-(9.3), and (c) AMF-CMC4-(9.3), immobilized onto cellulose surfaces.

degree of substitution. In addition, electrostatic repulsion of immobilized AMF-CMC with a higher DS_{COONa} can be a factor preventing higher deposition rates of additional AMF-CMC. It has to be considered that AMF has a pK_a value of 6.3 for the phenolic OH group.[38] Coupling of AMF to CMC does therefore not reduce the charge of the CMC backbone since one carboxylic group is replaced by one AMF molecule. The affinity of other AMF-CMC molecules to surfaces already bearing CMC is reduced and less AMF-CMC is deposited. These experiments clearly point out that AMF coupling has an influence on the immobilization efficiency on the cellulose films. However, the immobilization principle is not only based on the coupling of AMF since it could be shown in Figure 5 that CMC-EDC mixtures are deposited to a significant extent on cellulose surfaces.

Besides the degree of substitution, the EDC concentration, and the presence of AMF, the molecular weight of the polymers is another factor influencing the amount of deposited material. Compared to low molecular weight CMC1 (M_w: 90 kDa; DS_{COONa} 0.7, section 2.4.1, Figure 4) high molecular weight CMC2 (M_w: 250 kDa) of the same DS_{COONa} yields lower rates of immobilization (Figure 6). An explanation for this can be the fact that the accessibility of carboxylic groups is lower at higher molecular weights. This was also observed by Ogamio et al., who achieved only low degrees of AMF coupling with high molecular weight hyaluronic acid.[39]

The bulky conformation of already immobilized high molecular weight CMC will also lead to lower amounts of further immobilized material on the surface. A saturation of CMC is achieved faster if high molecular weight CMC is deposited.[14] Moreover, when the charge is sufficiently screened by substitution and cross-linking, low molecular weight AMF-CMC can easily coil when the electrostatic repulsion is reduced. Coiled molecules adsorb significantly better according to Kontturi et al. who suggested that low molecular weight polysaccharides are able to deposit in higher amounts onto cellulose surface.[40] The CMC with smaller dimension occupies less surface area on cellulose in the beginning of the deposition, thereby leaving more free space for further adsorption.[41] The

higher rate of deposition is also reflected in higher dissipation energy losses, which implies a higher water content.

The studies on the immobilization of purified AMF-CMC conjugates and reaction mixtures were conducted at neutral pH and without any additional electrolyte except EDC. It is known that the deposition of CMC on cellulose surfaces is favored at higher ionic strength of bivalent cations and at lower pH values.[14,16,42] Even though not studied in detail throughout this work, pre-experiments showed that an acidic pH increased the coupling efficiency of AMF to CMC and simultaneously enhanced the rate of deposition on cellulose surfaces. This allows controlling the rate of coupling and deposition, by adjusting the pH value and electrolyte concentration, which is especially of interest for pH sensitive biomolecules. In fact, the immobilization is governed not only by the activation and conjugation but also by the solution state of the CMC molecule, which can be influenced by the ionic strength and the pH value.

Immobilized isolated purified products and in situ immobilized CMC layers showed exceptional stability after rinsing with water at different pH values (3, 7, or 9) for 8 h. The time dependent frequency and dissipation changes of in situ immobilized layers of AMF-CMC1-(9.3) which were rinsed with water at different pH values are shown Figure 7. The

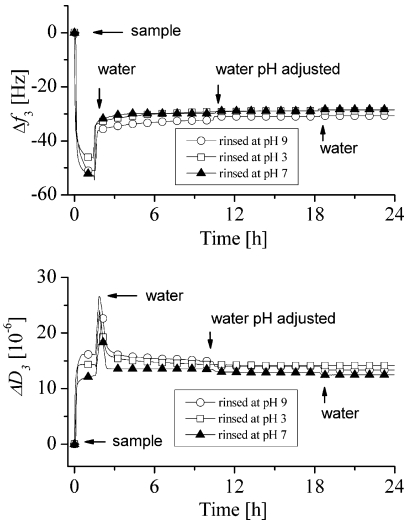


Figure 7. Changes in frequency and dissipation over time after the in situ deposition of AMF-CMC1-(9.3) on cellulose and subsequent rinsing with water at different pH values (3, 7, 9).

rinsing over time causes minor shifts in dissipation and frequency, indicating exceptionally stable layers. The layers are in addition stable against rinsing with SSC buffer containing a surfactant (data not shown). The method of immobilization presented here is therefore suitable for creating strongly and irreversibly bound layers on cellulose surfaces.

2.5. Topography of CMC Conjugate Modified Cellulose Surfaces. The cellulose surfaces, which were incubated with the reaction mixtures in the QCM-D experiments, were analyzed by AFM. The AFM image (1×1 μm^2) of a pure cellulose film shows a fiber-like structure after drying at ambient air at room temperature (Figure 8).

Cellulose films that were incubated with CMC1 and the reaction mixture CMC1-(9.3) result in a slightly modified surface. Nevertheless structural features similar to untreated cellulose model film are present. These changes are due to

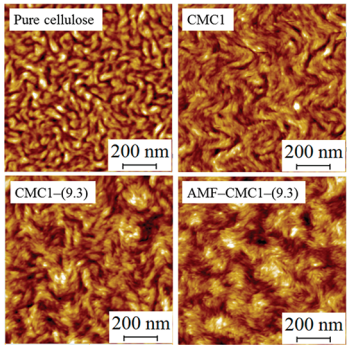


Figure 8. AFM topography images (1×1 μm^2) of a pure cellulose film and cellulose films from the QCM-D experiments treated with reaction mixtures of CMC1, CMC1-(9.3), and AMF-CMC1-(9.3). *Z*-scale, 20 nm.

minor surface coverage with CMC molecules or a molecular rearrangement of the hydrated cellulose layer. Both pure CMC1 and CMC1-(9.3) reaction mixtures were deposited in low amounts on the cellulose surface. This is in accordance with the QCM-D data.

The successful immobilization of AMF-CMC1-(9.3) onto a cellulose film results in a full surface coverage. The conjugate is deposited in such a high amount, that the fibril like structure of the base layer is no longer visible. A homogeneous distribution of the conjugates on the film can be seen, which further confirms the irreversibility of the immobilization after rinsing with water.

2.6. Microarrays of AMF-CMC Conjugates on Cellulose Films. QCM-D and AFM data suggested that reaction mixtures of AMF-CMC can be successfully immobilized on cellulose thin films. In order to apply this immobilization method in the preparation of sensor microarrays, the AMF-CMC reaction mixtures were microarray spotted onto cellulose coated microscope slides. Figure 9 shows fluorescent microarrays of immobilized AMF-CMC conjugates. Strong fluorescent spots can be observed. In contrast, the spots of AMF-CMC-(0), or mixtures of EDC and AMF, did not give any fluorescence signal after washing with water (images not shown). This implies again that AMF is bound to CMC in the presence of the conjugation agent EDC and that the conjugates

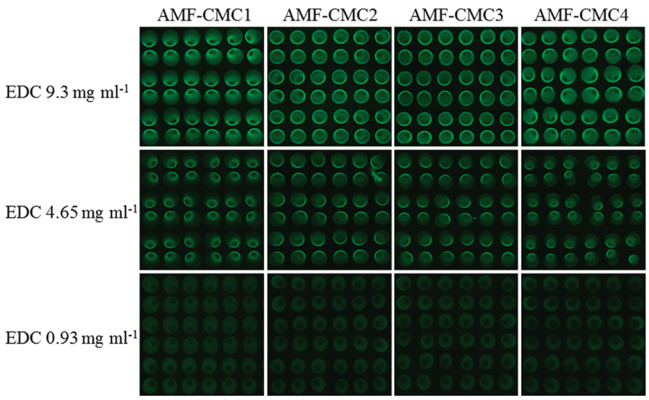


Figure 9. Fluorescence images of microarrays fabricated by the immobilization of AMF-CMC conjugates on solid cellulose. Lines: amounts of EDC in the reaction mixture. Columns: CMCs with different molecular weight and degree of substitution. Diameter per spot ~40 μm.

are irreversibly deposited on cellulose surfaces. Strong fluorescence signals are observed with the highest amounts of EDC (9.3 mg mL^{-1}) in the reaction mixture whereas reduced amounts of EDC also decrease the signal intensity of the immobilized spots. This is in accordance with the UV−vis spectroscopy and QCM-D data.

Interestingly high molecular weight AMF-CMC conjugates (CMC2 to CMC4) show an improved spot morphology compared to low molecular weight CMC. The higher molecular weight seems to improve the film forming behavior of the conjugates and leads to an evenly distributed material on the cellulose film.

It is worth noting that the spots exhibit exceptional stability against rinsing with water. This is advantageous with respect to the general applicability of the immobilization method. The deposition on other surfaces (pure COP and glass) did not give satisfying results with respect to the spot morphology and stability of the layers. The combination of CMC with a cellulose surface seems to be the crucial factor to obtain highly irreversible rates of immobilization.

Conjugation with other functional molecules bearing amino groups offers the possibility to immobilize these molecules on cellulosic surfaces. Further investigations in the pH and electrolyte dependent deposition rate and spot morphology are an ongoing topic of our research.

3. CONCLUSION

We successfully immobilized aminofluorescein conjugated carboxymethyl cellulose (CMC) onto cellulose surfaces. The conjugation of aminofluorescein with CMC was achieved with the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl). Purified, water-soluble conjugation products could be isolated and characterized. It was demonstrated that the degree of fluorescein conjugation to carboxymethyl cellulose strongly depends on the amount of carbodiimide added. Larger amounts of carbodiimide led to higher coupling rates. Quartz crystal microbalance measurement showed that the isolated and purified CMC conjugates can successfully be immobilized on cellulose surfaces by a simple incubation procedure. Furthermore it was shown that the conjugates can also be deposited in situ without isolation and purification. The in situ deposition of CMC conjugates depends on the degree of substitution, the amount of carbodiimide, the molecular weight of the CMC, and to a minor extent on the coupled aminofuorescein. Low molecular weight CMC with a low degree of substitution is irreversibly deposited at higher rates. The deposition was further confirmed by atomic force microscopy, which revealed a homogeneously distributed immobilized layer on cellulose surfaces. The knowledge obtained from these basic interaction studies was applied in microarray spotting. CMC conjugates were successfully spotted on cellulose coated microscope slides. The spot morphology was influenced by the molecular weight of CMC and the amount of carbodiimide. The versatile method develop in this work could further be extended to the conjugation of other functional molecules and their immobilization on cellulose and the selective modification of cellulosic materials in general.

AUTHOR INFORMATION

Corresponding Author

*E-mail: rupert.kargl@uni-graz.at (R.K.). Address: Karl-Franzens-University Graz Institute of Chemistry Heinrichstraße 28/III A-8010 Graz, Austria. Phone: 0043 316 380 5413. Fax: 0043 316 380 9850.

Notes

The authors declare no competing financial interest.

⊥Member of the European Polysaccharide Network of Excellence (EPNOE).

ACKNOWLEDGMENTS

The research leading to these results has received funding from the European Community's Seventh Framework Programme [FP7/2007-2013] under grant agreement no. 214015. Matej Bračič from the University of Maribor is highly acknowledged for the potentiometric charge titration measurements.

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